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13. SUPPLEMENTARY NOTES Effective 06/01/13, Dr. Roh has transferred to Northwestern University. Northwestern University, 1801 Maple Ave. 2nd Floor, Suite 2410. Evanston, IL 60201-3149					
14. ABSTRACT: Our main goal was to test the therapeutic value of PLK1 knockdown in combination with prostate cancer drugs in Pim-1 overexpressing cells. We hypothesized that depletion of PLK1 will result in synthetic lethality in Pim-1 overexpressing cells. We further hypothesized that depletion of PLK1 will further sensitize Pim-1 overexpressing cells to prostate cancer drugs. Our specific aims were to test whether loss of PLK1 can result in synthetic lethality in Pim-1 overexpressing cells and to test whether the loss of PLK1 can synergistically sensitize Pim-1 overexpressing cells to prostate cancer drugs. To date, we have found that loss of PLK1 reduced cell viability in general and this effect is more severe in Pim1 over expressing cells, suggesting synthetic lethality of Plk1 depletion and Pim-1 over expression. We found that Pim1-overexpressing cells are more prone to mitotic arrest followed by apoptosis. Interestingly, inhibition of PLK1 either by shRNA or BI 2536 in prostate cancer cell lines results in a reduction in MYC protein levels, and an increase in p53 levels. We also found that PLK1 depletion impairs the <i>in vivo</i> tumorigenicity of Pim1-expressing cells. Using Plk1 inhibitor, BI 2536, we confirmed these finding. Furthermore, we also found that PLK1 and PIM1 are frequently co-expressed in human prostate tumors using human tissue microarray samples.					
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INTRODUCTION

Intensive efforts are ongoing to explore novel targets and strategies for the management of prostate cancer. Our ultimate goal is to identify new molecules target(s) that can specifically sensitize Pim-1 overexpressing prostate cancer cells. Pim-1 is highly overexpressed in prostate cancer and overexpression of Pim-1 leads to genomic instability (Dhanasekaran et al., 2001; Roh et al., 2008; Roh et al., 2003) and docetaxel resistance in prostate epithelial cells (Zemskova et al., 2008). Using a siRNA library screen, we identified Polo-like kinase (PLK1) as a promising target whose knockdown can specifically reduce the cell viability of Pim-1 overexpressing cells. PLK1 is also overexpressed in a wide variety of cancer types including prostate and its expression correlates with poor patient prognosis (Strebhardt and Ullrich, 2006; Weichert et al., 2004). PLK1 has been an attractive molecular target for cancer therapy due to its structural hallmarks, its overexpression in various cancer types, and the intrinsic dependence of tumor cells on its activity in mitosis (Fink et al., 2007). Silencing of PLK1 has been shown to enhance drug sensitivity in some cancer cells such as pancreatic adenocarcinoma and breast (Spankuch et al., 2006; Yu et al., 2008). Our main goal is to test whether depletion of PLK1 will result in synthetic lethality in Pim-1 overexpressing cells and whether depletion of PLK1 will further sensitize Pim-1 overexpressing cells to prostate cancer drugs.

BODY

We will report on studies outlined in Aim 1 and Aim 2 of the proposal which cover Month 24-36.

Aim 1: To examine whether depletion of PLK1 results in synthetic lethality in Pim-1 overexpressing cells.

Task 2: We will examine the mechanistic basis of reduced cell viability of Pim-1 overexpressing prostate cells after PLK1 depletion by checking proliferation, apoptosis, cell cycle distribution and mitotic machinery. **Task 3:** We will test whether Pim-1 overexpressing cells lose tumorigenicity after PLK1 depletion by performing *in vitro* soft agar and *in vivo* xenograft assays.

Task, 2 & 3 Results: Previously, we have reported that Plk1 knockdown in Pim-1 overexpressing cells led to significantly reduced cell viability compared to Plk1 knockdown in Neo control cells, consistent with our original siRNA screen result in RWPE1 cells. We also reported that knockdown of PLK1 inhibited Myc expression, while increased p53 level and these results suggest a mechanistic relationship among these molecules. The reduced MYC expression seen in cells in which PLK1 is inhibited is particularly interesting in light of the known cooperativity between MYC and PIM1.

To explore additional mechanisms that could explain the reason why PIM1 sensitizes cells to the effect of PLK1 inhibition, we tested LNCaP-Neo and Pim1 cells with different dose of BI 2536. Pim1 cells were more sensitive to the effects of BI 2536; there are more mitotic arrested cells in Pim1 cells than Neo cells as shown by phosphor-specific histone H3 immunofluorescence (Fig. 1 A, B). Apoptotic marker, PARP showed that there are more apoptotic cells in Pim1 cells than Neo cells, especially at a lower dose of BI 2536 (10 nM) (Fig. 1 C). To avoid cell cycle-specific

effects, we synchronized these cells to G1/S phase using thymidine. We could detect more mitotic arrested cells and apoptotic cells in Pim1 cells than Neo cells shown by phosphor- H3 and PARP blotting (Fig. 1D). These data indicate that Pim1 cells are more sensitive to BI 2536 most likely due to increased mitotic arrest followed by apoptosis. Together our results suggest that Pim1 cells are hypersensitive to Plk1 inhibition due to increased mitotic arrest followed by apoptosis as well as reduced Myc protein levels upon Plk1 inhibition.

We next sought to determine if PLK1 depletion will impair the *in vivo* tumorigenicity of Pim1-expressing cells. We generated xenograft tumors using LNCaP-Pim1 cells and LNCaP-Neo cells with and without stable shPLK1 expression (Fig. 2). In this assay, we found that Pim1-expressing LNCaP cells with PLK1 knockdown formed significantly smaller tumors than the LNCaP-Pim1 controls (Fig. 2A). By contrast, no differences were observed between LNCaP-Neo and LNCaP-Neo/shPLK1 cells (Fig. 2A). This is probably because we selected cells with modest reduction in PLK1 to be able to discern the sensitizing effect of Pim1 expression and because cells with drastic knockdown of PLK1 are not viable.

To confirm our finding, we next employed Plk1 inhibitor, BI 2536. In this experiment, BI 2536 was given before tumor development (1 weeks post grafting). We found the modest inhibition of tumor growth in control LNCaP-Neo cells in BI 2536-treated mouse groups (Fig. 3A). In contrast, Pim1 tumors showed dramatic inhibition of tumor progression with a concomitant increase in mitotic index as shown by upregulation of histone H3 phosphorylation (Fig. 3 A-D). BI 2536 treatment increased apoptosis rate as shown by active caspase 3 staining in both in Neo and Pim1 cells with Pim1 cells showing more apoptosis (Fig. 4E, F).

Next, we addressed the question whether BI 2536 can still be effective once tumor developed. For this, we chose PC3-Pim1 cells and BI 2536 was administered after tumor developed (5 weeks 4 days post grafting). Tumor volume was dramatically reduced in both neo and Pim1 cells (Fig. 4H), however Pim1 cells showed more dramatic regression of tumor (average tumor volume: Pim1; 1089 mm³ to 380 mm³, Neo: 672.2 mm³ to 329 mm³).

In addition, we decide to examine whether Pim1 actually co-localize with PLK1 more frequently in clinical prostate tissue samples from patients diagnosed with PIN and carcinoma compared to normal prostate epithelium. To examine co-expression of PLK1 and PIM1 proteins in human prostate tumors, we employed immunohistochemical analysis of tissue microarrays (TMAs) from total prostatectomy specimens. Out of 162 specimens examined, PIM1 staining was observed in 77 (48%) cases and PLK1 staining was present in 55 (34 %) cases. There was considerable overlap between samples that express PLK1 and PIM1; 49 cases (30. 2%). In addition, co-expression of PLK1 and PIM1 was significantly correlated to higher Gleason grades. Out of 71 samples with Gleason grade 4-5, 25 (35.2 %) were PLK1/PIM1 double-positive, whereas 18 samples out of 54 samples with Gleason score 1-3 were double positive (33.3 %). PLK1 or PIM1 negative samples showed the opposite distribution; 32 out of 54 were Gleason grade 1-3 (59.3 %) and 14 out of 71 were Gleason grade 4-5 (19.7 %). These results support the idea that PLK1 and PIM1 are frequently co-expressed in human prostate tumors.

AIM 2: To test whether depletion of PLK1 further sensitizes Pim-1 overexpressing cells to prostate cancer drugs.

Task 1. We will test whether loss of PLK1 can synergize with prostate cancer drugs in Pim-1 overexpressing prostate cells.

Task 1 Result: Previously, we have reported that loss of Plk1 sensitized with doxorubicin in Pim-1 overexpressing cells in LNCaP cells. Using Plk1 inhibitor, BI 2536, we confirmed that Pim1 overexpressing cells are sensitive to Plk1 inhibition and BI 2536 and Pim-1 overexpression have synergistic effects on doxorubicin response in LNCaP cells.

Task 2. We will test whether loss of PLK1 can synergize with prostate cancer drugs in Pim-1 overexpressing prostate cells.

Task 2 Results: Nothing to report.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that increased mitotic cell arrest, apoptosis, and lower level of Myc upon Plk 1 inhibition in Pim 1 cells.
- Demonstration that Pim1 overexpressing prostate cancer cells are more sensitive to the inhibition of PLK1 by shRNA than control cells.
- Demonstration that Pim1 overexpressing prostate cancer cells are more sensitive to the effects of Plk1 inhibitor, BI2536 than control cells.
- Demonstration that PLK1 and PIM1 are frequently co-expressed in human prostate tumors.

REPORTABLE OUTCOMES

Abstract of manuscript in preparation:

PIM1 kinase is overexpressed in many tumor types including lymphomas and prostate cancer, where it is known to cooperate with the MYC oncogene in promoting tumorigenicity. PIM1 enhances MYC stability and transcriptional activity. Knowledge of the vulnerabilities of Pim1-expressing tumor cells will be of great value in efforts to develop novel anti-cancer therapeutics. Here we have used RNAi screening to identify genes whose depletion is detrimental to Pim1-overexpressing cells. We screened a collection of 570 siRNAs targeting cell cycle, apoptosis, serine-threonine kinase and tyrosine kinase genes for their effects on the viability of Pim1-expressing RWPE1 prostate epithelial cells. We identified the mitotic regulator polo-like kinase (PLK1) as a gene whose depletion is particularly detrimental to the viability of Pim1-expressing prostate cancer cells *in vitro* and *in vivo*. Inhibition of PLK1 by shRNA or by the chemical inhibitor BI2536 in prostate cancer xenograft model resulted in a dramatic inhibition of tumor progression in Pim1-overexpressing cells. Interestingly Pim1-overexpressing cells are more prone to mitotic arrest followed by apoptosis. Furthermore, inhibition of Plk1 activity led to the reduction of MYC protein levels both *in vitro* and *in vivo*. These results suggest that PLK1

inhibition may be particularly effective against PIM1-expressing tumors due, at least in part, to the fact that PLK1 inhibition reduces MYC protein levels.

CONCLUSION

Previously, we have reported that Plk1 knockdown in Pim-1 overexpressing cells led to significantly reduced cell viability compared to Plk1 knockdown in Neo control cells, consistent with our original siRNA screen result in RWPE1 cells. To understand possible mechanisms that could explain the reason why PIM1 sensitizes cells to the effects of PLK1 inhibition, we focused on mitotic arrest and apoptosis as well as MYC and p53. We found that Pim1-overexpressing cells are more prone to mitotic arrest followed by apoptosis. Interestingly, inhibition of PLK1 either by shRNA or BI 2536 in prostate cancer cell lines results in a reduction in MYC protein levels, and an increase in p53 levels. To determine if PLK1 depletion will impair the *in vivo* tumorigenicity of Pim1-expressing cells, we generated xenograft tumors using LNCaP-Pim1 cells and LNCaP-Neo cells with and without stable shPLK1 expression. Our results indicate that PLK1 depletion impairs the *in vivo* tumorigenicity of Pim1-expressing cells. Using Plk1 inhibitor, BI 2536, we confirmed these finding. Furthermore, we also found that PLK1 and PIM1 are frequently co-expressed in human prostate tumors using human tissue microarray samples. Going on, we will explore possible interaction and phosphorylation of PLK1 and Pim1. Tumorigenicity of PLK1 loss in combination with prostate drugs in Pim-1 overexpressing cells will be tested.

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APPENDICES

None

SUPPORTING DATA

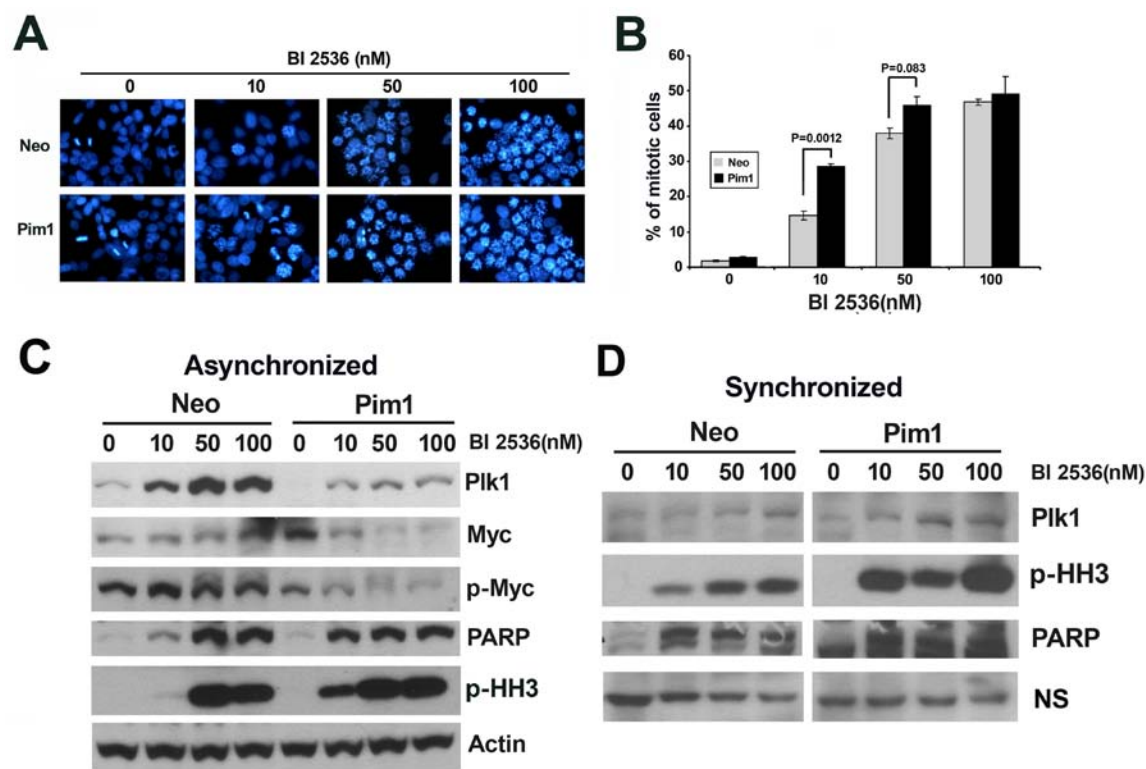


Fig. 1. Increased mitotic cell arrest, apoptosis, and lower level of Myc upon Plk 1 inhibition in Pim 1 cells. There are more mitotic arrested cells in Pim1 cells than Neo cells as shown by phosphor- specific histone H3 immunofluorescence (A,B) and immune blotting (C, D) upon BI 2536 treatment(C, D). Apoptotic marker, PARP showed that there are more apoptotic cells in Pim1 cells especially in lower dose of BI 2536 (10 nM) than Neo cell (C, D). Myc expression levels were also lower in Pim1 cells after BI2536 treatment (C).

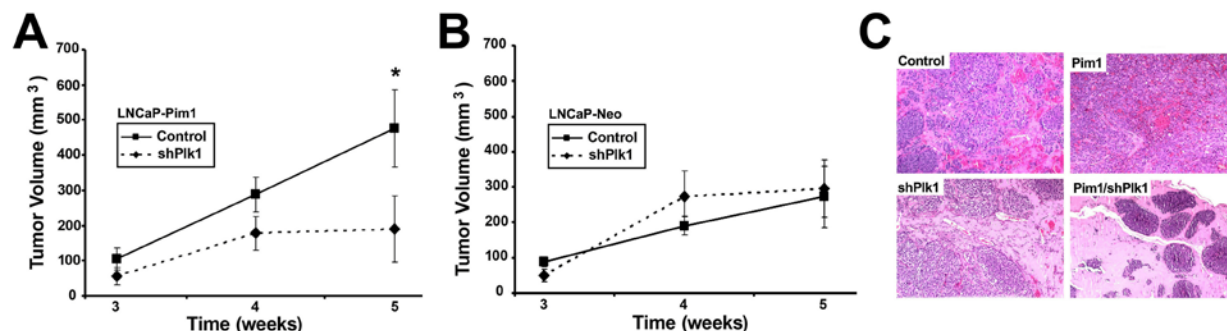


Fig. 2. Pim1 overexpressing LNCaP cells are more sensitive to the PLK1 knock-down. (A-B). Plk1 knock-downed LNCaP-Pim1 cells (Pim1/Plk1shRNA) as well as control shRNA knock-downed LNCaP-Pim1(Pim1/control shRNA) cells were injected into flank of nude mice and mean tumor volumes of each group 6 weeks after inoculation were shown. 10^7 cells per site were used. Pim1 cells formed big tumor, whereas Pim1/PlkshRNA cells formed significantly small tumor than Pim1 cells. Neo cells formed similar size of grafts regardless of shRNAs, Plk1shRNA and control shRNA. (C) H&E images of representative grafts from each group.

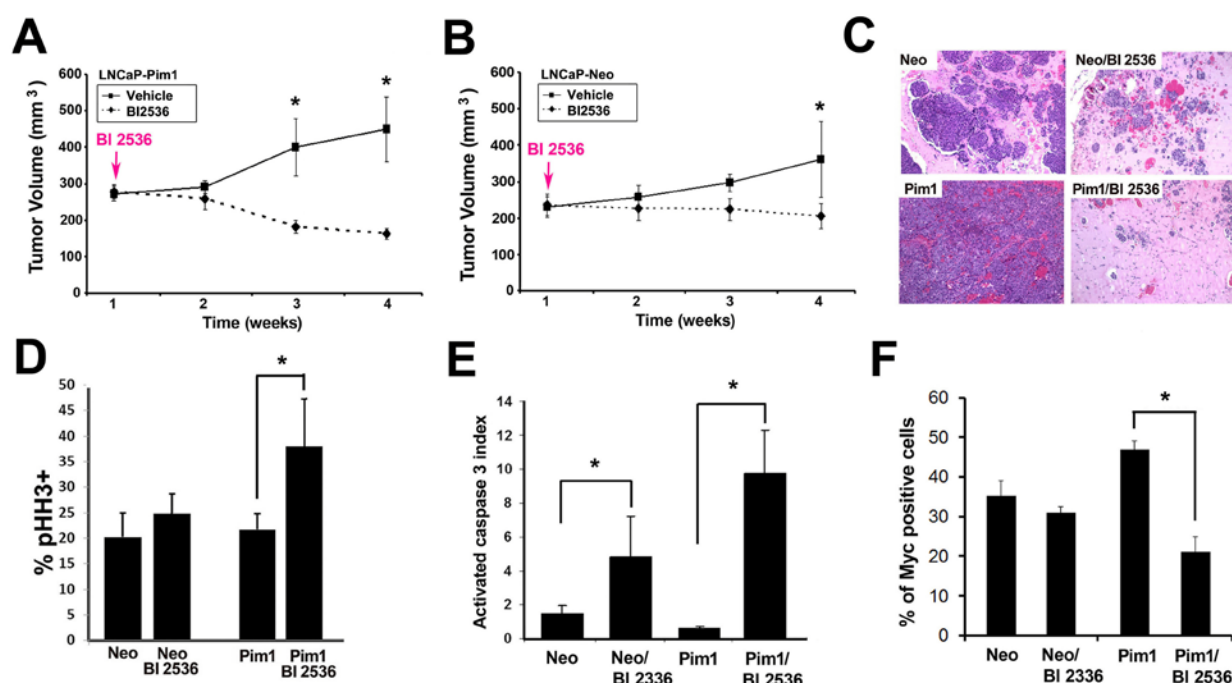


Figure 3. Pim1 overexpressing LNCaP cell are more sensitive to the effects of Plk1 inhibitor, BI2536. (A-B) LNCaP-Neo/Pim1 cells were grafted subcutaneously onto nude mice and one week later BI 2536 were injected intravenously at a dose of 25 mg/kg twice a week on two consecutive days for three weeks. $n = 10$ per group. Tumor size was measured once a week and mean tumor volume \pm SD are shown. Tumor volume was dramatically reduced in BI 2536-treated Pim1 cells. (C) Representative H&E images of each group. Notice massive tumor in Pim1 cells and dramatic reduction of tumors in BI 2536-treated Pim1 cells (Pim1/BI 2536). Neo cells also formed tumor and BI 2536 treatment led to the reduction of tumor, but Pim1 cells were

more sensitive to BI 2536 treatment. (D) Effective mitotic arrest after BI 2536 treatment in Pim1 cells as shown by quantitation of phosphor- histone H3 positive cells, whereas control Neo cells were less affected. (E) Increased apoptotic rate in BI 2536 treated mice. Quantitation of active caspase 3 positive cells is shown. (F) Quantitation of Myc immunostaining. Both Neo and Pim1 cells showed strong Myc positivity, whereas BI 2536-treated Pim1 cells showed much less Myc staining.

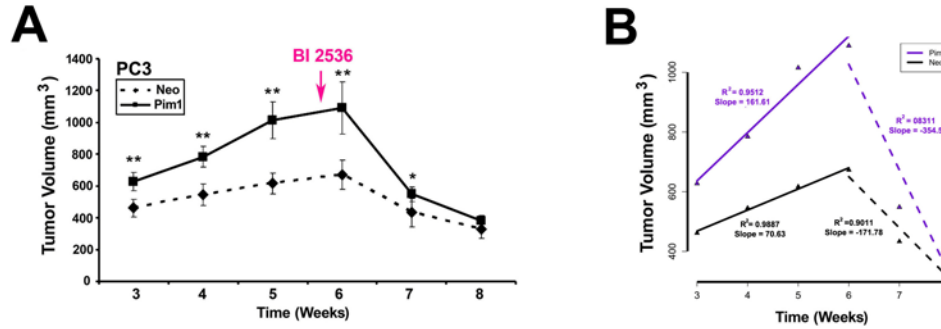


Figure 4. Pim1 overexpressing PC3 cell are more sensitive to the effects of Plk1 inhibitor, BI2536. (A) Tumors were induced by using PC3-Pim1 and -Neo cells and then mice were treated with BI 2536 i.v. for 6 cycles at a dose of 25 mg/kg twice a week starting from 5.5 weeks after grafting. n=10 per group. Mean tumor volume \pm SD are shown. Tumor volume was reduced in both Neo and Pim1 cells (Fig. 4H), however Pim1 cells showed more dramatic regression of tumor. (B) Graph showing the change of tumor volume over the time course. Notice the sharp decrease of tumor volume in BI 2536-treated Pim1 cells.

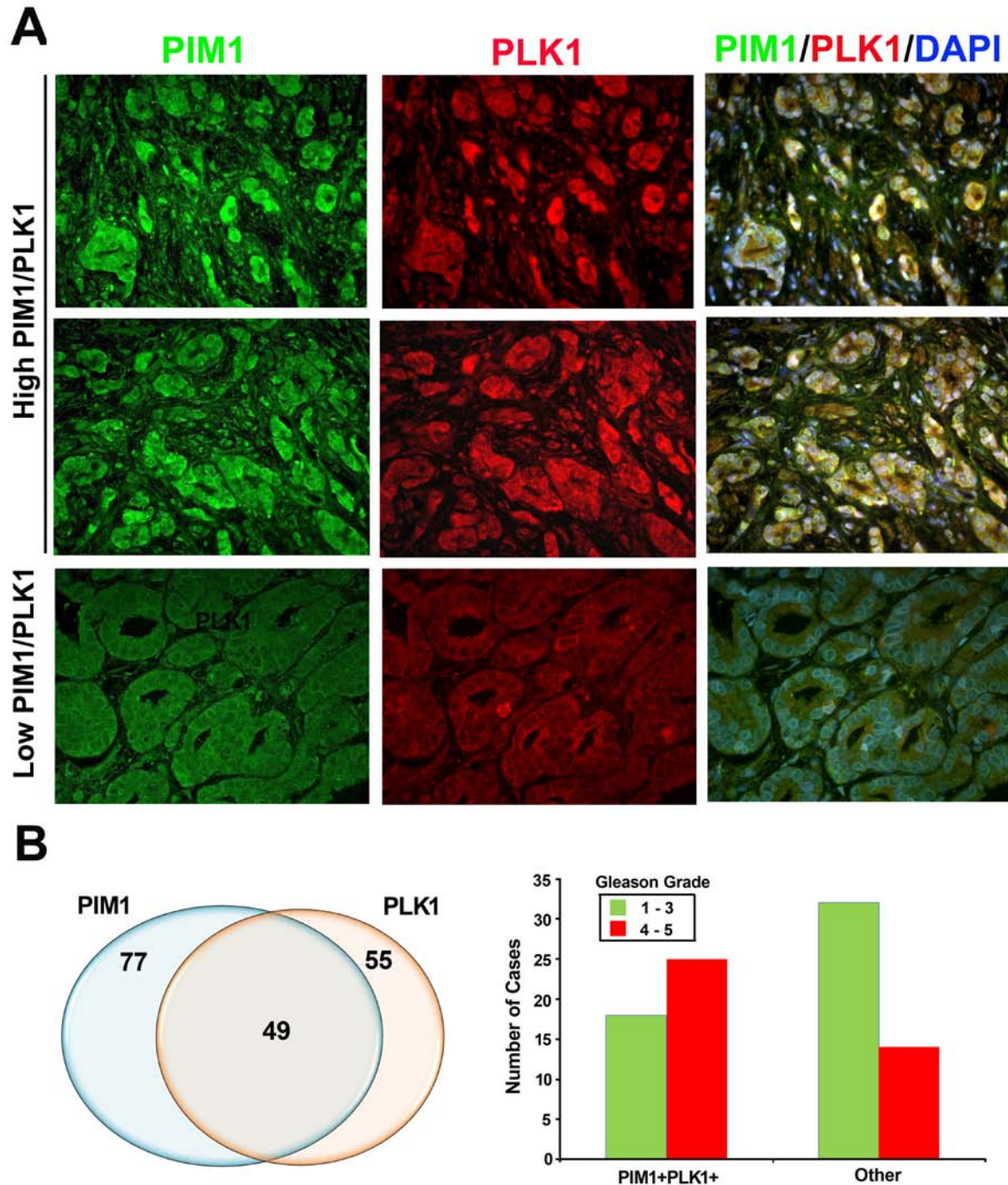


Figure. 5. Co-expression of PLK1 and PIM1 in human prostate tumors. (A) Representative immunofluorescence images of tissue microarrays from prostatectomy specimens. Out of 162 specimens examined, PIM1 staining was observed in 77 cases and PLK1 staining was present in 55 cases. There was considerable overlap between samples that express PLK1 and PIM1; 49 cases. (B) Co-expression of PLK1 and PIM1 was significantly correlated to higher Gleason grades.